PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 1/18, 33/536, 33/553, 33/537, 33/538, 33/533 — A1

(11) International Publication Number: WO 95/08105

(43) International Publication Date: 23 March 1995 (23.03.95)

(21) International Application Number: PCT/US94/10501

(22) International Filing Date: 16 September 1994 (16.09.94)

(30) Priority Data:

08/122,977	17 September 1993 (17.09.93)	US
08/238,686	5 May 1994 (05.05.94)	US

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(71) Applicant: SYSTEMIX, INC. [US/US]; 3155 Porter Drive, Palo Alto, CA 94304 (US).

(72) Inventors: READING, Christopher; P.O. Box 60203, Palo Alto, CA 94306 (US). CONDINO, Janet, L.; 638 Paco Drive, Los Altos, CA 94024 (US).

(74) Agent: LEHNHARDT, Susan, K.; Morrison & Foerster, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(54) Title: METHOD FOR OBTAINING A CELL POPULATION ENRICHED IN STEM CELLS AND COMPOSITIONS DERIVED THEREFROM

(57) Abstract

The invention provides methods for obtaining populations of cells enriched in stem cells utilizing a combination of physical cell separation; lysosomotropic agents and additional positive and negative purification steps. The invention further provides a population enriched in stem cells obtained by the methods described herein.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

METHOD FOR OBTAINING A CELL POPULATION ENRICHED IN STEM CELLS AND COMPOSITIONS DERIVED THEREFROM

Technical Field

This invention is in the field of isolation of cell populations enriched in hematopoietic stem cells.

Background Art

Mammalian hematopoietic (blood) cells provide a diverse range of physiological activities. Blood cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid lineages, comprising B cells and T cells, provide for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, and megakaryocytes as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

All publications cited herein are hereby incorporated herein by reference in their entirety.

Despite the diversity of the nature, morphology, characteristics and function of the hematopoietic cells, it is presently believed that these cells are derived from a single progenitor population, termed "stem cells." Stem cells are capable of self-regeneration and may become lineage committed progenitors which are dedicated to differentiation and expansion into a specific lineage.

A highly purified population of stem cells is necessary for a variety of *in vitro* applications and *in vivo* indications. Stem cells find use in a variety of clinical settings including, but not limited to, regenerating the hematopoietic system of a host deficient in stem cells; re-engrafting stem cells in a chemically

and/or radiation stem cell depleted host; producing various lineages of hematopoietic cells; detecting and evaluating growth factors relevant to stem cell self-regeneration; developing hematopoietic cell lineages; and assaying for factors associated with hematopoietic development. Stem cells are also a target for gene therapy to endow blood cells with novel properties.

Cell populations highly enriched in stem cells are essential for hematopoietic engraftment in cancer patients. Stem cells are important targets for gene therapy, where the genes inserted into the stem cells promote the health of the individual into whom the stem cells are transplanted. In addition, the ability to isolate stem cells may serve in the treatment of lymphomas and leukemias, as well as other neoplastic conditions. Thus, there have been world-wide efforts toward isolating human hematopoietic stem cells in substantially pure or pure form.

Stem cells constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are identifiable by the presence of a variety of cell surface protein antigen "markers." Such markers may be either specific to a particular lineage (lineage specific) or progenitor cell or be present on more than one cell type. Currently, it is not known how many of the markers associated with differentiated cells are also present on stem cells. One marker which was previously indicated as present solely on stem cells, CD34, is also found on a significant number of lineage committed progenitors. In particular, 80-90% of the $CD34^+$ population is marked by lineage specific and non-specific markers.

Table 1 summarizes probable phenotypes of stem cells in fetal, adult, and mobilized peripheral blood. In Table 1 myelomonocytic stands for myelomonocytic associated markers, NK stands for natural killer cells and AMPB stands for adult mobilized peripheral blood. As

used herein both infra, supra and in Table 1, the negative sign or, uppercase negative sign, (⁻) means that the level of the specified marker is undetectable above Ig isotype controls by fluorescence activated cell sorter (FACS) analysis, and includes cells with very low expression of the specified marker.

Table 1

Probable Stem Cell Phenotypes

	NK and T cell markers			B cell markers			Myelomonocytic			Other							P-gp Activity
	CD2	CD3	CD8	CD15	CD19	CD20	CD14	CD15	CD19	CD33	CD34	CD38	HLA-DR	C-Kit	Thy	Rho	
FBM	-	-	-	-	-	-	-	-	-	?	+	-	+	+	+	lo	+
ABM	-	-	-	-	-	-	-	+	-	-	+	?	lo/-	+	+	lo	+
AMPB	-	-	-	-	-	-	-	-	-	lo/-?	+	?	lo/-	?	+	lo	+

In view of the small proportion of the total number of cells in the bone marrow or peripheral blood which are stem cells, the uncertainty of the markers associated with stem cells as distinct from more differentiated cells, and the general inability to assay for human stem cells biologically, the identification and purification of stem cells has been elusive. Characterizations and isolation of human hematopoietic stem cells are reported in: Baum et al. (1992) Proc. Natl. Acad. Sci. USA 89:2804-2808; and Tsukamoto et al. U.S. Patent No. 5,061,620, as identifiable by the presence of CD34 and Thy-1 but lacking lineage specific markers ($CD34^+Lin^-$ $Thy\text{-}1^+$).

Current methods to isolate and purify cell populations rely on a combination of negative and positive selection techniques. Negative selection is a process which depletes specific cell types to yield a cell population more highly concentrated in the population to be purified. Negative selection is usually insufficient to yield a suitable purified population of cells and must be followed by positive selection. Positive selection directly isolates the cells to be purified on the basis of cell specific characteristics. Positive selection techniques include, but are not limited to, FACS.

Effective negative selection techniques lower the quantity of cells which must be put through the FACS, which has limits of about 3,000 cells per second. Red blood cells must be removed by negative selection prior to FACS as they impair FACS by altering light scatter patterns. Granulocytes and monocytes also seriously hamper FACS sorting by clumping and clogging the sorter. Also, throughput greater than 3,000 cells/second is necessary to obtain clinically significant amounts of stem cells.

Physical or mechanical separation techniques are also used to separate different hematopoietic cells and

to isolate stem cells. Counter-flow centrifugal elutriation (elutriation) separates on the basis of sedimentation velocity. Elutriation can fractionate cells into three groups: lymphocytes; monocytes and stem cells; and granulocytes, although there is a great deal of cross-contamination due, at least in part, to heterogeneity of sedimentation velocity within a cell type. For a review of the use of elutriation as a negative selection method see Takaue et al. (1986) Am. J. Hematol. 23:247-262. Further physical separation techniques include, but are not limited to, magnetically stabilized fluidized bed such as that described in United States patent application serial No. 08/130,094. Other types of separation techniques utilize equilibrium buoyant density sedimentation to separate cell types and cellular debris on the basis of their respective buoyant densities. Such separation techniques include, but are not limited to, centrifugation in Ficoll or Percoll.

FACS can also be used to separate cells on the basis of physical characteristics. The type of light scatter of particular cells can be used to identify these cells for separation purposes. Low forward light scatter indicates small elements such as platelets and red blood cells. Forward light scatter increases with increasing cell size: lymphocytes, stem cells, monocytes, and granulocytes in that order. Side scatter is an indication of internal structure. Granulocytes, which have high forward scatter because of their large size, also have high side scatter due to intracellular granules. Red blood cells, having little internal structure, have low side scatter as well as low forward scatter.

Characteristics other than cell surface markers are also used to isolate stem cells. Decreased rhodamine 123 (rho123) staining of hematopoietic cells is determined not by the initial dye accumulation but by an efflux process sensitive to P-glycoprotein (P-gp) inhibitors.

Retention of several P-gp-transported fluorescent dyes, including rho123, in human bone marrow cells was found to be inversely correlated with the expression of P-gp. Bone marrow cells expressing physical and antigenic characteristics of pluripotent stem cells showed high levels of P-gp expression and fluorescent dye efflux. Fractions of human bone marrow cells isolated on the basis of either increased rho123 efflux or P-gp expression contained practically all the primitive progenitor cells of human bone marrow, including LTC-initiating cells. Chaudhary and Roninson (1991) Cell 66:85-94. The use of a variety of separation methods is described in Reading and Takaue (1986) Biochim. et Biophys. Acta 865:141-170.

Lower alkyl esters of L-amino acids are known to be lysosomotropic agents. The mechanism of action of these agents involve their diffusion into the lysosomes of monocytes and granulocytes where the ester linkage is cleaved to yield the zwitterionic amino acid residue which cannot diffuse out of the lysosome. The resulting osmotic imbalance ruptures the lysosomal membrane, releasing degradative enzymes, which cause lysis of the cells. Monocytes lyse immediately, whereas granulocytes do not.

Lysosomotropic agents such as L-phenylalanine methyl ester hydrochloride (PME) have been shown to lyse human monocytes in vitro and have been used to selectively decrease the proportion of monocytes in mixtures of leukocytes. Thiele et al. (1983) J. Immunol. 131:2282-2290; Rubenstein and Ballow (1989) Clin. Lab. Immunol. 30:35-39; Rosenfeld et al. (1990) Blood 76:1783-1787; and Law et al. (1990) Exp. Hematol. 18:582. PME has been found to deplete monocytes and phagocytic cells without abolition of colony-forming ability in the remaining cells. Chau and Law (1991) Int. J. Cell Cloning 9:211-219. Lysosomotropic agents have been shown to deplete monocytes and natural killer cells. Thiele and Lipsky

(1985) J. Immunol. 134:786-793; Thiele and Lipsky (1985) Proc. Natl. Acad. Sci. USA 82:2468-2472; Thiele and Lipsky (1986) J. Immunol. 136:1038-1048; Thiele et al. (1987) J. Immunol. 138:51-57; Borrebaeck et al. (1988) Proc. Natl. Acad. Sci. USA 85:3995-3999; Raff et al. (1988) Transplantation 46:655-660; Thiele and Lipsky (1990) Proc. Natl. Acad. Sci. USA 87:83-87; Blazar et al. (1990) Blood 75:798-805; Thiele and Lipsky (1990) J. Exp. Med. 172:183-194; Pecora et al. (1991) Transplantation 51:524-531; Townsend and Simon (1992) J. Immunother. 12:256-264; and U.S. Patent Nos. 5,206,221, 5,068,223, 5,047,401 and 4,752,602.

PME has been reported to be a "fugitive" reagent, selectively lysing monocytes without destroying the cytolytic function of lymphokine-activated killer cells (LAK cells) or NK cells. Hoyer et al. (1986) Cancer Res. 46:2834. Certain L-amino acid lower alkyl esters have been found to increase the biological activity of LAK cells. Leung (1989) Cancer Immunol. Immunother. 30:247-253; Wersäll et al. (1990) Acta Oncol. 29:431-437; Foon et al. (1992) J. Immunother. 11:184-190; and U.S. Patent No. 4,849,329.

Elutriation cannot be immediately used for negative selection of white blood cells from peripheral blood or bone marrow because of the high red blood cell content of these tissues. The standard practice is to dilute the sample and subject it to equilibrium density gradient centrifugation on a Ficoll-diatrizoate density gradient. The mononuclear cells, defined as a cell population containing hematopoietic cells as well as other cells with a single nucleus, band at the interface of the density gradient medium and the sample. The red blood cells and the mature granulocytes, having greater densities by virtue of hemoglobin iron and dense granules respectively, sediment to the bottom of the tube. The remaining cells can then be further separated by other methods including, but not limited to, FACS, elutriation

or affinity chromatography. Flow sorting of cells is described by Lindmo et al. (1990) Flow Cytometry and Sorting, 2 ed. Wiley Liss, Inc.

Recently, mouse stem cells have been obtained in at least highly concentrated, if not a purified form, where fewer than about 30 cells obtained from bone marrow were able to reconstitute all of the lineages of the hematopoietic system of a lethally irradiated mouse. Each assayed cell is multipotent for all blood lineages, while self-renewal is variable amongst these cells. Spangrude et al. (1988) Science 241:58-62; Smith et al. (1991) Proc. Natl. Acad. Sci. USA 88:2788-2792; Uchida Ph.D. Thesis. Stanford U. (1992); and see also, EPA 89.304651.6 and the references cited therein which describe the isolation of mouse stem cells.

The routine use of stem cells for patient therapy awaits a system that can isolate large amounts of highly purified stem cells within a clinically relevant time frame. Few stem cell purification systems provide such a system.

Summary of the Invention

The invention provides methods for obtaining a cell population enriched in hematopoietic stem cells utilizing physical separation, the addition of at least one lysosomotropic agent, at least one purification step to remove cell debris and residual red blood cells, and a purification step to sort specifically for cells expressing stem cell specific markers but not lineage specific markers. The invention further provides a cell population enriched in stem cells obtained by the methods described herein.

Modes for Carrying Out the Invention

The present invention provides a method for obtaining a cell population substantially enriched in stem cells. As used herein, stem cells are those

hematopoietic cells expressing stem cell specific markers but not lineage specific markers. The method results in a high yield of stem cells which are capable of differentiating into the various hematopoietic lineages associated with stem cells and thus are not merely progenitor cells.

The method comprises the steps of obtaining a hematopoietic cell population substantially free of red blood cells, physically separating the cells under conditions effective to obtain a cell population enriched in monocytes and stem cells, treating this cell population with at least one lysosomotropic agent at a concentration and under conditions sufficient to deplete monocytes and granulocytes and to retain stem cells, removing cell debris, and sorting the processed cells so as to obtain those cells expressing stem cell specific markers but not lineage specific markers.

Hematopoietic cells can be obtained from a variety of sources including, but not limited to, adult bone marrow, adult peripheral blood, adult mobilized peripheral blood fetal bone marrow, fetal liver, fetal peripheral blood, umbilical cord blood and cadaveric bone marrow. Methods of obtaining these cells are known in the art and need not be described in detail herein. Although the invention is described with reference to human hematopoietic cells, it is to be understood that the invention applies to the corresponding cells of other mammals. Preferably, the invention is used with human cells and for the treatment of humans. Preferably, adult mobilized peripheral blood is used.

The hematopoietic cells should be substantially free of red blood cells. Preferably, substantially free of red blood cells means that the hematocrit is less than 5%. More preferably, the hematocrit is less than 2% of the cells. A peripheral blood preparation may be collected using a blood cell processor such as the Fenwall CS-3000 Cobe Spectrum or Haemoneties USO. At the

time the blood is obtained from these instruments, it may be substantially free of red blood cells and no further processing may be necessary prior to initiating the method described herein. Further processing may be done while the blood is being collected or after collection. In the case of bone marrow cells, a processing step is generally required to remove excess red blood cells. Any method known in the art is suitable for this purpose including, but not limited to, equilibrium buoyant density sedimentation. Suitable methods are described in the Examples. Preferably, the method performed on the Stericell as described in the Examples is used.

The hematopoietic cell population substantially free of red blood cells is then subjected to physical separation. Any method known in the art that is not lethal to stem cells is suitable for use. Suitable methods include, but are not limited to, elutriation and magnetically stabilized fluidized bed. Preferably, elutriation is used. Methods of performing physical separation are known in the art and are not described in detail herein. Elutriation is described in the Examples.

Physical separation takes place under conditions effective to obtain a cell population enriched in monocytes and stem cells. Generally, the monocytes are present at about 30 to 50% of the enriched cell population. Stem cells are a small percentage of the original cell population which varies according to the initial concentration. Preferably the absolute number of stem cells is not less than 0.5% of the total original number of cells. More preferably, the absolute number of stem cells is not less than 5% of the total original number of stem cells. Methods of determining the concentration of monocytes and stem cells are known in the art.

The physically separated cell population is then treated with a lysosomotropic agent. Such agents include, but are not limited to, L-amino acid lower alkyl

esters and hydrogen chloride salts thereof. Lower alkyl means an alkyl group of 1-4 carbon atoms. Carbon atoms include, but are not limited to, methyl and ethyl groups. L-amino acid lower alkyl esters suitable for use in the present invention are those which cause lysis of monocytes but which do not substantially affect viability of stem cells. Preferably, the reagents also cause lysis of granulocytes. Preferably, the reagents are relatively stable such that they do not form toxic compounds when placed in solution.

Preferably the reagent used is L-phenylalanine methyl ester (PME). Although L-leucyl leucine methyl ester (LLME) is described in the literature as useful in lysing monocytes it is generally not preferred for use in the present invention. LLME forms toxic polymers when placed in solution at concentrations over 0.05 mM. These polymers are toxic to all cell types and thus lyse not only monocytes but stem cells as well. Several L-amino acid lower alkyl esters are described in the art as not being particularly toxic to monocytes. These are L-isoleucine-methyl-ester, L-valine methyl ester, L-alanine methyl ester, L-glycine methyl ester and D-leucine methyl ester. Thiele and Lipsky (1985) J. Immunol. 134:786-793. These authors also describe L-leucine-methyl-ester, L-leucine-ethyl-ester and L-glutamic acid methyl ester as being effective in lysing monocytes. Given the discussion and examples herein and known cell identification methods, it is within the skill of one in the art to determine which L-amino acid lower alkyl esters are effective to deplete monocytes and granulocytes and retain stem cells.

It is preferable to incubate the cells with the lysosomotropic agent under conditions sufficient to substantially deplete any granulocytes that may be present. This is because the kinetics of cell death differ for granulocytes and monocytes. Monocytes lyse fairly rapidly and completely in the presence of

lysosomotropic agents. Granulocytes, having fewer lysosomes, undergo a slower cell death in response to lysosomotropic agents. Granulocyte death involves the formation of vacuoles and change in cell shape preceding cell lysis. Should further purification steps continue prior to lysis of granulocytes, the lysed cells can cause cell clumping and the unlysed cells cannot be properly sorted due to the changes in cell morphology and internal structure.

The incubation conditions effective to lyse monocytes and granulocytes must be determined empirically for each lysosomotropic agent and are within the skill of one in the art. In the case of PME, the ideal reaction conditions have been found to be 5 mM PME final concentration, incubation at room temperature for forty minutes and then at 37°C for 30 minutes. These conditions are optimal, a variety of other conditions are satisfactory. For instance, incubations at 4°C overnight and at room temperature for 2 hours and then at 4°C overnight yield results comparable to the optimal conditions. It is necessary to use the most rapid method for obtaining stem cells for clinical use. Therefore, if the cells are to be used in clinical treatment, the method described in the Examples is preferred.

Lysis of monocytes and granulocytes results in a great deal of cell debris which must be removed prior to continued cell processing. Any method known in the art is suitable for use including, but not limited to, equilibrium buoyant density sedimentation. In practice, a Percoll separation is performed essentially according to the manufacturer's instructions. In this instance, the cells form a pellet and the cellular debris floats on the surface of the Percoll solution. The supernatant can be aspirated to yield a cell pellet enriched in stem cells.

A step may be included to deplete the cell population of substantially any remaining mature red

blood cells. This step may be performed by equilibrium density gradient banding of mononuclear cells or by incubation of cells with ammonium chloride.

A final, sorting step then follows to yield cells expressing stem cell specific markers but not lineage specific markers. Any method known in the art is suitable for use including, but not limited to, FACS. Preferably the cells are sorted for expression of CD34 and lack of lineage specific markers ($CD34^+Lin^-$). Lineage specific markers include, but are not limited to, CD10, 19, 20, SIg for B-cells; 16, 33 for myeloid; 15 for granulocytes, 14 for monocytes, 41 for megakaryocytes, 38 for lineage dedicated, CD3, 4, 7, 8, TCR for T-cells and glycophorin for erythroid. More preferably, the cells also express the Thy-1 antigen ($CD34^+Thy\text{-}1^+Lin^-$). Prior to FACS sorting, the cells are stained with fluorescent antibodies specific for the particular markers.

The following examples are provided to illustrate but not limit the invention.

EXAMPLES

Summary: The following procedure is used to isolate stem cells from hematopoietic cell populations derived from either peripheral blood or bone marrow. For peripheral blood samples with a hematocrit of greater than 5%, the cell samples are sequentially subjected to FACS, Ficoll purification, elutriation, PME lysis, ammonium chloride lysis, and FACS sorting. For peripheral blood samples with a hematocrit of less than 5%, the cell samples are sequentially subjected to FACS, elutriation, PME lysis, Ficoll flotation and FACS. For bone marrow samples, the cell samples are sequentially subjected to FACS, StemCell™ purification for banding of mononuclear cells by equilibrium density sedimentation, elutriation, PME lysis, and FACS sorting.

Example 1

FACS Analysis Procedure

FACS analysis was performed according to standard methods known in the art using antibodies specific for CD34, Thy-1, and lineage specific markers (14^-, 15^-). Briefly, the following procedure was used. For FACS staining and analysis, 300 μL of each cell sample is used. The remainder of the cell samples were reserved for the isolation of stem cells.

1) A 3-well stain in a microtiter plate is used for STAT analysis. Into each of 3 wells, add 100 μL of cells by pipette. Centrifuge plate at 350 x g for 3 minutes and aspirate off the supernatant.

2) Add 50 μL of diluted stain (either CD34, Lin (14^-, 15^-), or Thy specific antibodies) to each well. Incubate on ice for 20 minutes.

3) Wash wells with 150 μL of 1.5 x PBS with 1% HSA (staining buffer). Centrifuge at 350 x g for 5 minutes and aspirate off the supernatant.

4) Resuspend each cell pellet in 50 μL of appropriate second antibody stain mixture. Cover from light, incubate on ice for 20 minutes.

5) Wash wells as specified in Step 3.

6) Resuspend in 300 μL of propidium iodide (PI) (1:1000 in staining buffer) per well.

Samples are analyzed using a dual laser set up for fluorescein isothiocyanate (FITC) / phycoerythrin (PE) / Texas Red (TR) system.

Example 2

Semi-automated Isolation of Mononuclear Cells Using the StériCell™

Initial purification of bone marrow was performed as follows. Bone marrow was obtained by aspiration and the white blood cells were counted. 1×10^8 cells are dispensed into labeled tubes and diluted to 20 mL with BW medium (BioWhittaker Elutriation Media with 0.5% HSA, pH

7.2). The platelets, red blood cells and plasma were removed from the sample using the StériCell™ Processor (DuPont) according to the manufacturer's instructions and the fraction between 1.040 and 1.079 g/mL was reserved for subsequent purification steps.

Example 3

Elutriation

The following describes two sets of procedures: one used to elutriate cells for clinical purposes and a second procedure used to elutriate cells for research purposes (on a smaller scale). Research scale elutriation is performed on a Beckman Model # J6MI, while the chamber used for clinical scale elutriation is an enlarged version of the Sanderson chamber (Beckman instruments PN 356945 (small chamber), PN 354397 (large chamber)) which has a volume approximately 6.7 times greater than that of the small Sanderson chamber. For clinical use of the cells obtained, all parts of the elutriater that come in contact with the cells must be sterilized.

CLINICAL ELUTRIATION

Cleaning and Sterilization:

Before assembly, the chamber, chamber gasket, and all components of the Quick-Release Assembly which come into contact with the sample are sonicated for two minutes in a 1% solution of Liquinox and rinsed exhaustively with deionized water. The black seal is checked for scratches and irregularities, and, if necessary, polished by hand. The Quick-Release Assembly is assembled according to the manufacturer's directions with new O-rings and new bearings being used in the assembly. The chamber is assembled according to the manufacturer's directions with the chamber screws untightened. The Quick-Release Assembly and the chamber are autoclaved separately.

Assembly of Elutriation System

In a biosafety cabinet or laminar-flow hood, unwrap the Chamber Assembly and tighten the chamber screws with the hex wrench provided by the manufacturer. Unwrap the Quick-Release Assembly and remove the sterile gauze covering the end of the two short tubes. Install the chamber on the Quick-Release Assembly according to the manufacturer's directions.

Open the semi-closed Harness packet and pull out the inlet and outlet ends to a length of about 18 inches. Dock the appropriate ends to the inlet and outlet tubing of the Quick-Release Assembly using the SCD. Install the Quick-Release Assembly in the rotor body and attach the anchor cable. Remove the rest of the Semi-closed Harness from the packet and lay it out in the biosafety cabinet. Pass the inlet and outlet lines under the appropriate arms of the cable retainer and over the back chamber gasket. Install the bubble chamber and position the tubing in the pump head. Close the pump head. Spike into the bag of elutriation buffer and hang the bag.

Pump Calibration

Open Clamp 1 on the Semi-closed Harness; check to make sure all other clamps are closed. Start the pump at a setting of 1.00. Visually check the Quick-Release Assembly and all connections for leaks. Turn the centrifuge on and set the speed for 2000 rpm. Close the lid and start the rotor spinning. While the rotor is accelerating, check the level of liquid in the bubble trap; if it goes to the red line, it is an indication of excessive back pressure in the bubble trap (usually caused by air in the chamber). With the pump still going, stop the rotor and allow the air to be pumped out. Re-start the rotor. Place Bag #1 on the balance and tare it. Check to make sure that the pump is at 1.00, and pump for 1 minute. Record the weight and multiply by 1.004 g/mL to obtain the volume delivered at this

setting. Repeat at pump settings 2.00 and 3.00 to obtain a calibration curve. Enter the pairs of values obtained into the HP11C according to the manufacturer's directions for Linear Regression. This will provide pump settings for the flow rates used for clinical elutriation:

FRACTION	SPEED	FLOW RATE	COLLECT TIME
Load	2500 rpm	15 mL/min	13′
E1	2000 rpm	25 mL/min	10′ 30"
E2	2000 rpm	53 mL/min	7′
E4	R/O		10′

Elutriation

Set the rotor speed to 2500 rpm and the pump to the LOAD setting. Turn on the pump and start the centrifuge. While the rotor is accelerating, check to make sure Clamp 1 is open and Clamps 2 and 3 are closed. After the rotor has reached the set speed, open the sample clamp to begin introducing sample into the chamber. Start the timer. When the sample bag is empty, open the buffer clamp and close the sample clamp. At the end of 13′, increase the flow rate to the pump setting for E1, Reset the timer. At the end of 10′30" open Clamp 2 and close Clamp 1. At the end of 7′, turn off the rotor, open Clamp 3 and close Clamp 2. Reset the timer. At the end of 10′, turn off the pump and, using the Sebra Tube Sealer, disconnect the exit portion of the Semi-closed Harness from the Quick-Release Assembly. Using the Sebra Tube Sealer, remove Bag 2 leaving the entire connector on the bag. Pellet the cells at 350 x g for 20 minutes.

Research Elutriation

1. Cleaning, Bearing and Seal Check

Once a week, or oftener if it appears necessary, disassemble the separation chamber and the rotating seal. Using the ultrasonic cleaner with 0.1-0.2% Liquinox or other appropriate laboratory detergent sonicate the chamber, chamber gasket, both short tubes and the

transfer tube for 2 minutes. Rinse well with deionized (DI)H_2O.

Check the bearings by spinning by hand; if one or both "feels" rough, replace it (them). Both bearings do not have to be replaced at the same time. Lap the rotating seal and check the height of the raised rings (see Operation Manual). If the height of the rings is less than 1/16", or if the seal has radial scratches or is chipped, replace it and order a new one at the first opportunity.

2. Assembly System

Track assembly with checklist provided in Operation Manual. On the bench, assemble the chamber for single chamber elutriation (single dots on top and bottom of chamber face up). Assemble the rotating seal. Place a SMALL amount of Spinkote under the chamber mounts to facilitate sliding of the chambers, and attach the chamber and rotating seal (in that order) to the central plate. Double check rotor assembly: While the Quick Release Assembly is still on the bench, inject about 20 mL of water into the inlet port using a syringe with a short piece off #16 tubing. If the water doesn't come out the outlet port, check the position of the single dots on the chamber. Install a Quick Release Assembly in rotor body. Connect inlet and outlet tubing to their respective ports. Check to make sure that all components external to the rotor, bubble trap, pressure gauge, pump, sample valve, and tubing, are present and in the correct position. See diagram provided in Operation Manual.

3. Sterilization

If the rotor is already clean and assembled, this will be your starting point for a separation. From this point on, work should be done in the hood if a sterile product is necessary. If you are working in the Nuaire hood, turn on the blower and wait about 15 minutes.

Close the pump head and, with a setting of about 1, pump 500 mL of 10% sodium hypochlorite (household bleach)

through the system. Do this with the bubble trap upside down so that it fills with bleach. Make sure the bubble trap/pulse suppressor and the chamber are completely filled with bleach solution. Hold the Quick Release Assembly upright so that the air exits. Turn the bubble trap upside down.

Follow the bleach solution with 500 mL of sterile water and 500 mL of sterile saline or 1 L of sterile saline, again making sure that the bubble trap and the chamber are filled with liquid during this step. When about 125 mL of sterile saline remains in the bottle, calibrate the pump (Section 5).

4. Sample Preparation

Obtain a cell count on the sample. Dilute 20 μL sample with 180 μL of saline. The target range for number of cells to introduce into the large Sanderson chamber is about $5 \times 10^9 - 1 \times 10^{10}$. The cell number is total cells, white cells plus red cells.

5. Pump Calibration

Have ready a 50 mL graduated cylinder, a timer, stopwatch, and a calculator with a linear regression program. The pump should be calibrated while the rotor is at run speed. Turn the centrifuge on if you have not already done so and, if necessary, enter the correction speed and temperature. Set the time for 59 hours, 59 minutes. Check to see that the Quick Release Assembly is installed correctly and that there are no large air bubbles in the chamber. Set the pump speed to 1.00 using the 10-turn helipot on the front of the pump and turn the pump on. Make sure the pump is pumping in the correct direction, i.e., from the reservoir to the rotor. If it is not, switch pump directions using the toggle switch on the pump. To avoid blowing a fuse on the pump, go from one pump direction to the other with a slight pause in the middle.

Start the rotor spinning by pressing ENTER, START. When the rotor reaches 2000 rpm, using a 50 mL, sterile,

graduated cylinder, collect effluent for one minute at settings of 1.00, 1.50 and 2.0. Using a linear regression program, determine the pump settings which will produce desired flow rates. In the case of peripheral blood and human bone marrow, the preferred parameters are ROTOR SPEED 2000 rpm and TEMPERATURE 22°C.

FRACTION # LOAD	FLOW RATE SMALL CHMBR	FRACTION VOLUME	FLOW RATE LARGE CHMBR (15 mL/min at 2500 rpm)	APPROXIMATE FRACTION VOLUME (= 335 μL)
1	9.6 mL/min	100 mL	25 mL/min	
2	14 mL/min	100 mL	64 mL/min	Collected together at 53 mL/min
3	16 mL/min	100 mL	73 mL/min	
4	R/O	100 mL	R/O	

Alternatively, the following parameters are used; loading is at 15 ml/min at 2500 rpm and then the flow rates for the large chamber at 2000 rpm are: E1, 25 ml/min; E2 includes E2 and E3 from small chamber, 53 mL/min; and E3, R/O.

USING LINEAR REGRESSION PROGRAM IN HP11C

Turn on, press + to clear, press 1, then ENTER. Enter flow rate obtained at pump set of 1.0. Repeat these two steps for the other pump set/ flow rate pairs. To determine the pump set for a given flow rate, enter the flow rate and press f then ŷ,r. This calibration will last so long as the pump rollers are not released or the position of the tubing on the rollers is not otherwise changed.

6. Sample Reservoir and Elutriation Media

After the sterile saline has been pumped through, turn off the pump and remove the old syringe barrel on the sample valve and replace it with a new, sterile one. A 10 mL syringe is a good size, but any size which is appropriate for your sample volume is fine.

Pour about 10 mL of sterile elutriation media into the sample reservoir. Without touching the pipette end on the inlet line, place it in the bottle of elutriation media (0.9% NaCl, 0.1% dextrose, 0.3 mM EDTA from BioWhittaker).

7. Bubble Trap/Pulse Suppressor

To adjust the height of the liquid in the bubble trap, clamp it in the upright position. Turn the sample valve so that air is being pumped into the line through the sample reservoir. When media in the bubble trap sinks to a height of about 20 mm, turn the sample valve so that it is again pumping media.

8. Final Preparation

Number eight 50 mL capped, conical centrifuge tubes, 2 each, #1-#4, and place them in the circular rack on the pump. Fill ice bucket. Restart the rotor and set the pump speed at the setting corresponding to the flow rate for Fraction 1. Check the pressure gauge; if it is greater than 2-3, there is some air in the chamber. To flush out the air, stop the rotor while the pump is still pumping.

9. Separation

Pour the sample into the sample reservoir. Place the end of the outlet tube in the empty sample tube. Turn the sample valve to start the sample flowing into the rotor while collecting the effluent. When the level of liquid in the sample reservoir is near the bottom, pour the effluent collected in the sample tube into the reservoir. Transfer the outlet line to the first fraction collection tube. When the level of liquid in the sample reservoir is at the bottom, turn the valve so that media is being pumped from the reservoir. If you forget to turn the valve, the bubble trap will allow for some air. If too much air is pumped into the system, the bubble trap will be depleted and air will be pumped into the rotor. A large increase in back pressure will ensue, and it will be necessary to abort the run.

When a large amount of air gets into the chamber, back pressure will increase, often to the point where it will be necessary to abort the run. If this happens, making sure the outlet is in a 50 mL test tube, shut off the rotor while maintaining (or increasing) flow rate. All of the cells will exit into the test tube. Fill the chamber with media and start up the rotor again. Continue to collect from the effluent line while doing this in order to minimize cell loss. Readjust the flow rate to collect Fraction 1 and reintroduce the sample.

When 50 mL has been collected in the first tube, move the outlet line to the second tube and collect an additional 50 mL at the first flow rate. Move the outlet line to the first tube numbered 2 and increase the flow rate to collect Fraction 2. It does not matter whether you move the outlet tube and then increase the flow rate or vice versa. Just do one or the other consistently. Repeat the previous step until all fractions have been collected. When collecting the last fraction, turn the pump up to 2.0 and turn off the rotor. This will flush out the contents of the rotor. Making sure all tubes are capped securely, pellet the cells at 350 x g for 17 minutes.

10. Shut-down

Pump about 500 mL of dilute lab detergent (0.1% Liquinox) through the system followed by 500 mL of de-ionized H_2O. Finally flush the system with about 50 mL ETOH.

11. Fractions

Collect the samples in 8 tubes, 2 tubes per fraction. Aspirate off the supernatant and resuspend the cells, combining cells from the first and second tube of each fraction. Use 5 - 10 mL of HBSS plus 5% HSA or other appropriate media to resuspend cells.

In the case of larger volumes of cells for sorting, the following procedure may be used.

Elutriation

Set the rotor speed to 2500 rpm and the pump to the LOAD setting. Turn on the pump and start the centrifuge. While the rotor is accelerating, check to make sure Clamp 1 is open and Clamps 2 and 3 are closed. After the rotor has reached the set speed, open the sample clamp to begin introducing sample into the chamber. Start the timer. When the sample bag is empty, open the buffer clamp and close the sample clamp. At the end of 13', increase the flow rate to the pump setting for E1. Reset the timer. At the end of 10'30", open Clamp 2 and close Clamp 1. At the end of 7', turn off the rotor, open Clamp 3 and close Clamp 2. Reset the timer. At the end of 10', turn off the pump and, using the Sebra Tube Sealer, disconnect the exit portion of the Semi-closed harness from the Quick-Release Assembly. Using the Sebra Tube Sealer, remove Bag 2 leaving the entire connector on the bag. Pellet the cells at 350 x g for 20 minutes.

Example 5

PME Lysis

The following procedure was used for lysis of monocytes and granulocytes. Cells from the elutriation procedure are suspended in MEM at a concentration of 2 X 10^7 cells/mL. PME lysis solution containing MEM, 2 mM NaOH, 1% HSA, 50 μg/mL DNase Type I, and 10 mM PME is prepared and sterilized immediately before use. 1 mL of the PME lysis solution is added to an equal volume of cell suspension (2 x 10^7 cells). The mixture is incubated at room temperature for 40 minutes and at 37°C for 30 minutes. "100% Percoll" is prepared by adding 9 parts Percoll liquid (Pharmacia) to one part 10 x PBS. A volume of this 100% Percoll sufficient for a final concentration of 25% is added to the PME incubation. The cells are pelleted by centrifugation at 914 x g for 20 minutes in a Beckman centrifuge model # GS6R, with a GH38 rotor.

Example 6

Ammonium Chloride Lysis

The following procedure was used to complete lysis of the cells obtained in Example 5. Resuspend the cell pellet obtained from centrifuging the PME lysis suspension in 25% Percoll in ammonium chloride buffer which has been kept at 4°C. Use 50 mL of lysis buffer for each 10^8 cells. Incubate the cells for 5 minutes on ice. Centrifuge at 600 x g for 5 minutes to pellet the cells. Aspirate off the supernatant.

Example 7

Staining Clinical Samples for Sorting

The following procedure was used to stain the cells obtained in Example 6 for subsequent FACS. The antibodies used correspond to commercially available antibodies. Resuspend the cells in Staining Buffer (MEM, 1% HSA, pH 7.4) containing Gammimmune (1:50) at a cell concentration of 2 x 10^7. Cells and reagents must be kept cold from this point on. Add PR3-Fab'2-SR (anti-CD34-sulforhodamine labeled), PR4-FITC, and PR9-FITC (anti-14,15). Anti-CD34 at 2.5 μg/mL was added at 6.5 μL/mL of cell suspension. The cell suspension in all cases was 2 x 10^7/mL. The lineage-specific antibodies were at 1 μg/mL each and were added at 2 μL/mL of cell suspension. The anti-Thy antibody was 5 μg/mL and used at 6.5 μL/mL of cell suspension. Remove two 50 μL aliquots for Gate tubes 1 and 2 Place tubes on ice covered with foil. Add PR13-b (biotin labeled) to a final concentration of 6.5 μg/mL to sample. Mix. Remove 50 μL aliquot for Gate tube 3. Place on ice covered with foil. Add IgG1-b (Biotin) to Gate tube 1 to a final concentration of 5 μg/mL. Incubate sample on ice in the dark for 20 minutes. Add 10 μL/mL of cell suspension streptavidin where the initial concentration of streptavidin is 150 μg/mL. Remove a 50 μL aliquot for Gate tube 4. Place on ice covered with foil. To Gate tubes 1 and 2, add 1 μL

streptavidin at the same concentration. Incubate Gate tubes 1 and 2 on ice in the dark for 20 minutes. Add Staining Buffer to the sample so that the final volume is 50 mL. Centrifuge at 600 x g for 5 minutes. Add 500 μL of Staining Buffer to each of the four Gate tubes; centrifuge at 1300 x g for 2 minutes. Aspirate off the supernatant from the sample and from the cells in the gate tubes. Repeat the wash. Resuspend the sample in Staining Buffer so that the final concentration is once again 2 x 10^7 and add biotin PE (bPE) at 10 μg/mL, used at 11 μL/mL cells. Resuspend the cells in Gate tubes 1-3 in bPE and the cells in Gate tube 4 in 50 μL Staining Buffer. Incubate all tubes on ice in the dark for 20 minutes. Dilute the sample tube to 50 mL with Staining Buffer and the Gate tubes with 500 μL of Staining Buffer. Centrifuge to pellet the cells. Resuspend the sample so that the cell concentration is 10^7/mL. Resuspend the cells in the Gate tubes with 500 μL of Staining Buffer.

Example 8

Staining for FACS Analysis

FACS was performed according to standard methods known in the art using antibodies specific for CD34, Thy-1, and lineage specific markers (14^-, 15^-). Briefly, the following procedure was used.

1) A 3-well stain in V-bottom microtiter plate is used for STAT analysis. Into each of 3 wells, pipette 100 μL (10^6) cells. Centrifuge plate at 350 x g for 3 min. Aspirate supernatant.

2) Add 50 μL of diluted stain to each well. Incubate on ice for 20 minutes.

3) Wash wells with 120 μL staining buffer. Centrifuge at 350 x g for 5 minutes, aspirate supernatant.

4) Add second antibodies as in example 1. Incubate 20 minutes on ice. (Keep in the dark from this point on). Wash wells as specified in Step 3.

5) Add 25 μL of IgG_1 block to all wells. Incubate 10 minutes on ice. Do not wash.

6) Resuspend each cell pellet in 50 μL of appropriate antibody stain mixture. Keep covered from light, incubate on ice for 20 minutes.

Resuspend in 300 μL of PI buffer (1:1000 in staining buffer) per well, as each sample is transferred to 12 x 75 FACS tubes. Cover with foil to shield from light and read on FACS sorter.

Example 9

Summary of PME Results

Table 2 summarizes the results obtained from a series of experiments performed as described in Examples 1-7. In Table 2, HN and BM stand for bone marrow; PB stands for mobilized peripheral blood; 34L is the percentage of $CD34^+Lin^-$ cells in the original sample; Tot34L is the actual number of $CD34^+Lin^-$ cells in the sample; rec34L is the percent recovery of the $CD34^+Lin^-$ cells; My is the percent mature myeloid cells in the original sample as determined by high orthogonal light scatter; TotMy is the actual number of these mature myeloid cells in the sample; DepMy is the percent depletion in the sample after the indicated series of steps; 34LT is the percentage of $CD34^+Thy\text{-}1^+Lin^-$ cells in the original sample; Tot34LT is the total number of $CD34^+Thy\text{-}1^+Lin^-$ cells in the original population; %R34LT is the percentage of $CD34^+Thy\text{-}1^+Lin^-$ cells obtained after the indicated step; the lower case "e" indicates that the indicated step was performed after elutriation.

TABLE 2

ID	Stage	Cell #	34L (%)	Tot34L	rec34L (%)	My (%)	TotMy	DepMy (%)	34L (%)	Tot34L	%R34LT
HN264	BM	1.00E + 09	0.45	4.48E + 06	100.00	68.00	6.80E + 08	0.00	0.03	3.14E + 05	100.00
930730	Stericell	2.90E + 08	1.44	4.16E + 06	92.89	42.60	1.24E + 08	81.83	0.11	3.20E + 05	102.18
	CD15	3.30E + 07	2.06	6.79E + 05	15.16	10.50	3.47E + 06	99.49	0.14	4.76E + 04	15.16
	PME	8.60E + 08	1.66	1.43E + 05	3.19	20.80	1.79E + 06	99.74	0.79	6.82E + 04	21.76
HN266	BM	1.40E + 09	1.51	2.12E + 07	100.00	67.10	9.39E + 08	0.00	0.39	5.53E + 06	100.00
930805	Stericell	3.40E + 08	1.46	4.96E + 06	23.42	61.60	2.09E + 08	77.70	0.26	8.68E + 05	15.70
	PME	8.31E + 07	7.96	6.61E + 06	31.21	31.40	2.61E + 07	97.22	2.76	2.29E + 06	41.50
HN289	Stericell	2.00E + 08	2.16	4.33E + 06	100.00	41.50	8.30E + 07	0.00	0.29	5.84E + 05	100.00
930819	PME	5.30E + 07	1.83	9.71E + 05	22.43	8.40	4.45E + 06	94.64	0.56	2.97E + 05	50.84
PB094.1	PBMC	1.30E + 09	1.04	1.35E + 07	100.00	88.10	1.15E + 09	0.00	0.51	6.62E + 06	100.00
930805	e2 + 3	5.00E + 08	1.39	6.96E + 06	51.74	78.90	3.95E + 08	65.55	0.64	3.18E + 06	48.05
24 hrs	PME	3.26E + 08	3.91	1.27E + 07	94.72	76.30	2.49E + 08	78.28	2.01	6.54E + 06	98.76
PB097.3	pre	1.30E + 09	0.24	3.15E + 06	100.00	82.70	1.08E + 09	0.00	0.18	2.11E + 06	100.00

TABLE 2 (Cont'd)

ID	Stage	Cell #	34L (%)	Tot34L	rec34L (%)	My (%)	TotMy	DepMy (%)	34L (%)	Tot34L	%R34LT
930810	E2+3	5.80E + 08	0.60	3.48E + 06	110.64	53.80	3.12E + 08	70.98	0.38	2.19E + 06	103.86
20 hrs	PME37.30	2.03E + 08	1.32	2.68E + 06	85.27	42.50	8.63E + 07	91.98	0.81	1.65E + 06	78.13
PB098.4	pre	1.30E + 09	0.51	6.57E + 06	100.00	61.60	7.94E + 08	0.00	0.22	2.81E + 06	100.00
930811	e2 + 3	5.00E + 08	1.01	5.03E + 06	76.51	49.70	2.49E + 08	68.71	0.54	2.71E + 06	96.18
	PME37.30	4.50E + 08	1.01	4.52E + 06	68.80	56.30	2.53E + 08	68.10	0.43	1.94E + 06	68.96
PB114.1	pre	6.00E + 08	0.13	7.92E + 05	100.00	67.00	4.02E + 08	0.00	0.06	3.45E + 05	100.00
930826	e123	2.20E + 08	0.07	1.53E + 05	19.33	91.30	2.01E + 08	50.03	0.04	9.19E + 04	26.67
20%HCT	postPME	1.10E + 08	6.04	6.65E + 06	839.06	17.90	1.97E + 07	95.10	3.15	3.46E + 06	>95
PB113.6	pre	6.80E + 08	2.85	1.94E + 07	100.00	84.60	5.75E + 08	0.00	0.77	5.27E + 06	100.00
930831	e123f	4.10E + 08	11.07	4.54E + 07	234.29	43.40	1.75E + 08	69.07	1.97	8.08E + 06	153.32
24 hrs	PME	6.00E + 08	11.07	6.64E + 06	34.28	36.40	2.18E + 07	96.20	2.06	1.24E + 06	23.44
PB113.7	pre	6.60E + 08	0.70	4.61E + 06	100.00	92.80	6.12E + 08	0.00	0.19	1.28E + 06	100.00
930901	ePME	3.80E + 08	12.04	4.58E + 07	991.95	43.20	1.64E + 08	73.20	0.37	1.42E + 06	111.01
24 hrs	eCD15	5.60E + 07	8.23	4.61E + 06	99.88	52.70	2.95E + 07	95.18	0.09	5.07E + 04	3.97

TABLE 2 (Cont'd)

ID	Stage	Cell #	34L (%)	Tot34L	rec34L (%)	My (%)	TotMy	DepMy	34L (%)	Tot34L	%R34LT
PB121.2	pre	6.20E + 08	1.11	6.85E + 06	100.00	77.90	4.83E + 08	0.00	0.57	3.53E + 06	100.00
930903	eCD15	9.60E + 06	16.49	1.58E + 06	23.11	6.30	6.05E + 05	99.87	6.53	6.27E + 05	17.77
12%hct	ePME	5.63E + 07	25.92	1.46E + 07	212.82	32.50	1.83E + 07	96.21	6.45	3.63E + 06	102.90
PB124.5	pre	5.70E + 08	2.92	1.66E + 07	100.00	68.30	3.89E + 08	0.00	0.73	4.19E + 06	100.00
930909	ePME1	1.10E + 08	10.71	1.18E + 07	70.85	16.00	1.76E + 07	95.48	2.93	3.23E + 06	77.03
PB124.6	pre	6.00E + 08	1.98	1.19E + 07	100.00	82.90	4.97E + 08	0.00	0.83	4.97E + 06	100.00
930910	ePME	1.5E + 08	19.02	2.85E + 07	239.68	32.10	4.82E + 07	90.32	2.09	3.14E + 06	63.07
24 hrs	eCD15	144.E + 07	11.82	1.70E + 06	14.31	50.50	7.27E + 06	98.54	1.38	1.99E + 05	4.00

Note that in some samples the original percentage of stem cells was very low. Generally, if there are less than 1% $CD34^+$ cells in the initial sample, the estimation of yields are approximate and subject to a high degree of error. It is clear from the results obtained that the present method yields a high percentage of the stem cells; in fact, close to 100% of the stem cells in the original sample can be recovered.

Example 10

Comparison of Bone Marrow Samples Subjected to PME Lysis and Those Subjected to CD15 Magnetic Bead Depletion

The CD15 bead depletion was performed by methods known in the art. Briefly, 15 mL of HBSS with 2% HSA was added to anti-CD-15 antibody coated magnetic beads (roughly four times as many beads as granulocytes in the bone marrow sample) and the tube was placed in the magnetic stand. After 2 to 3 minutes, the buffer was carefully removed without disturbing the beads. The tube was removed from the magnetic stand and this wash step was repeated three additional times. The beads were resuspended in 1 mL of HBSS with 2% HSA and the bone marrow cells were resuspended in the same buffer at a concentration of 2×10^7 cells/mL. 1 mL of cell suspension was mixed with 1 mL of bead suspension and incubated on ice for 30 minutes, gently rotating the tube every minute. The tube was placed in the magnetic stand and the beads allowed to collect on the sides of the tube. The non-bound cell fraction was removed. The beads were washed as before with 10 mL of HBSS with 2% HSA, the buffer was pooled with the previous non-bound cell fraction, and the CD15 bead depleted cells harvested by centrifugation.

The results presented in Table 2 indicate that compared to CD15 bead depletion, the method of the present invention yields a significantly higher

percentage of stem cells (111% v. 3.97%; 102.90% v. 17.77%; and 63.07% v. 4%).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. It should be apparent that one having ordinary skill in this art would be able to surmise equivalents to the invention as described in the claims which follow but which would be within the spirit of the description above. Those equivalents are to be included within the scope of this invention. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

CLAIMS

1. A method of obtaining a composition enriched in hematopoietic stem cells comprising the steps of:

a) obtaining a population of hematopoietic cells substantially free of red blood cells;

b) separating the cells by physical means under conditions effective to obtain a cell population enriched in monocytes and stem cells;

c) treating the physically separated cell population with at least one lysosomotropic agent at a concentration and under conditions sufficient to deplete monocytes and granulocytes and to retain stem cells;

d) removing cell debris from the treated cells; and

e) sorting the cells so as to obtain those cells expressing stem cell specific markers but not lineage specific markers.

2. The method according to claim 1 wherein the hematopoietic cell population is obtained from adult bone marrow, adult peripheral blood, adult mobilized peripheral blood, fetal bone marrow, fetal liver, fetal peripheral blood, neonate liver, neonate peripheral blood, umbilical cord blood or cadaveric marrow.

3. The method according to claim 1 wherein the hematopoietic cell population is human.

4. The method according to claim 1 wherein the hematocrit of the hematopoietic cell population is less than about 5%.

5. The method according to claim 1 wherein the hematocrit of the hematopoietic cell population is less than about 2%.

6. The method according to claim 1 wherein the hematocrit of the hematopoietic cell population is in the range of about 2% to 5%.

7. The method according to claim 1 wherein the hematopoietic cell population is obtained from adult peripheral blood.

8. The method according to claim 1 wherein the hematopoietic cell population is obtained from mobilized peripheral blood.

9. The method according to claim 1 wherein the hematopoietic cell population is obtained from bone marrow and is pre-processed to remove excess red blood cells.

10. The method according to claim 9 wherein the pre-processing step is equilibrium buoyant density sedimentation.

11. The method according to claim 1 wherein the physical separation is effected by elutriation.

12. The method according to claim 1 wherein the physical separation is effected by a magnetically stabilized fluidized bed.

13. The method according to claim 1 wherein the lysosomotropic agent is a L-amino acid lower alkyl methyl ester.

14. The method according to claim 13 wherein the lower alkyl has 1-4 carbon atoms.

15. The method according to claim 13 wherein the amino acid is phenylalanine.

16. The method according to claim 15 wherein the L-amino acid lower alkyl methyl ester is L-phenylalanine methyl ester.

17. The method according to claim 1 wherein the L-amino acid lower alkyl methyl ester is selected from the group consisting of L-leucine methyl ester, L-leucine ethyl ester and L-glutamic acid methyl ester.

18. The method according to claim 16 wherein the L-phenylalanine methyl ester is present at a final concentration of about 1 mM to 10 mM.

19. The method according to claim 18 wherein the L-phenylalanine methyl ester is present at a final concentration of 5 mM.

20. The method according to claim 16 wherein the incubation is at room temperature for about forty minutes and then at 37°C for 30 minutes.

21. The method according to claim 1 wherein the cell debris is removed by equilibrium buoyant density sedimentation.

22. The method according to claim 21 wherein the cell debris is removed by centrifugation in an effective concentration of Percoll.

23. The method according to claim 1 wherein the cell sorting is done by fluorescence activated cell sorting.

24. The method according to claim 1 wherein the cells are sorted on the basis of cell surface markers.

25. The method according to claim 24 wherein the stem cells express CD34 but not lineage specific markers.

26. The method of claim 25 wherein the stem cells further express Thy-1.

27. A composition comprising the cells obtained from step e) of claim 1.

28. A method of obtaining a composition enriched in hematopoietic stem cells comprising the steps of

a) obtaining a population of hematopoietic stem cells from mobilized peripheral blood substantially free of red blood cells;

b) separating the cells by elutriation under conditions effective to obtain a cell population enriched in monocytes and stem cells;

c) treating the physically separated cell population with at least one-phenylalanine lower alkyl ester at a concentration and under conditions sufficient to deplete monocytes and granulocytes and to retain stem cells;

d) removing cell debris from the treated cells; and

e) sorting the cells by fluorescence activated cell sorting so as to obtain those cells expressing stem cell specific markers but not lineage specific markers.

29. The method according to claim 28 wherein the hematocrit of the hematopoietic cell population is less than about 5%.

30. The method according to claim 28 wherein the hematocrit of the hematopoietic cell population is less than about 2%.

31. The method according to claim 28 wherein the hematocrit of the hematopoietic cell population is in the range of about 2% to 5%.

32. The method according to claim 28 wherein the lower alkyl has 1-4 carbon atoms.

33. The method according to claim 28 wherein the L-amino acid lower alkyl methyl ester is L-phenylalanine methyl ester.

34. The method according to claim 28 wherein the L-phenylalanine methyl ester is present at a final concentration of about 1 mM to 10 mM.

35. The method according to claim 34 wherein the L-phenylalanine methyl ester is present at a final concentration of 5 mM.

36. The method according to claim 33 wherein the incubation is at room temperature for about forty minutes and then at 37°C for 30 minutes.

37. The method according to claim 22 wherein the cell debris is removed by centrifugation in an effective concentration of Percoll.

38. The method according to claim 25 wherein the stem cells express CD34 but not lineage specific markers.

39. The method of claim 26 wherein the stem cells further express Thy-1.

40. A composition comprising the cells obtained from step e) of claim 28.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10501

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 1/18, 33/536, 33/553, 33/537, 33/538, 33/533
US CL :435/2, 7.24; 436/536, 526, 538, 541, 546, 172, 175, 177, 178
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 7.24; 436/536, 526, 538, 541, 546, 172, 175, 177, 178

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE
search terms: stem cells, lineage markers, DNA, hematopoietic, lysosomotropic, equilibrium buoyant density, elutriation, flow cytometry

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	American Journal of Hematology, Volume 23, issued 1986, Takaue et al, "Depletion of T Lymphocytes From Human Bone Marrow by the Use of Counterflow Elutriation Centrifugation", pages 247-262, see entire document.	11, 28-40
Y	US, A, 5,061,620 (TSUKAMOTO ET AL.) 29 October 1991, see entire document.	12, 26, 39
Y	International Journal of Cell Cloning, Volume 9, issued 1991, Chau et al, "Effect of L-Phenylalanine Methyl Ester on the Colony Formation of Hematopoietic Progenitor Cells from Human Bone Marrow", pages 211-219, see entire document.	17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 DECEMBER 1994	Date of mailing of the international search report 10 JAN 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY J. PARSONS Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10501

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	International Journal of Cell Cloning, Volume 7, issued 1989, Law et al, "Removal of Peripheral Blood Monocytes by Phenylalanine Methyl Ester Has No Effect on the Colony Growth of Hematopoietic Progenitor Cells", pages 100-110, see entire document.	1-40
Y	Journal of Experimental Medicine, Volume 174, issued November 1991, Peault et al, "Lymphoid Reconstitution of the Human Fetal Thymus in SCID Mice with CD34$^+$ Precursor Cells", pages 1283-1286, see entire document.	1-40